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A Highly Sensitive Array-Based POCT Sensor for Respiratory Pathogens

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(5 Pages Phase II Plan)

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PHASE I FINAL REPORT

1.0 WHAT ARE THE PROBLEMS?

Respiratory infection is the most common cause of acute infectious disease in U.S. adults, the leading cause of outpatient illnesses, and a major cause of hospitalization for U.S. military personnel. Because of crowded living conditions, stressful work environments, higher level of travel, and exposure to respiratory pathogens in disease-endemic areas, both military trainees and new troops are particularly at high risk for experiencing respiratory disease epidemics. Emeging respiratory pathogens, such as adenovirus, influenza virus, *Streptococcus pyogenes, Bordetella pertussis and Streptococcus pneumoniae* are particularly problematic.[1-2] Respiratory infections are generally diagnosed by: culture isolation of viruses from clinical specimens, serologic tests and enzyme-linked immunosorbent assay (EIA) for rises in specific antibodies in serum samples, and fluorescent-antibody (FA) staining on clinical specimens.[3-6] Culture identification takes several days or weeks to produce final results. Serodiagnosis is done with acute-phase and convalescent-phase serums collected at an interval of 2 to 4 weeks, requiring special equipment and biological laboratory skills. The complement fixation and Enzyme Immunoassay (EIA) are tests for group-specific (genus-reactive) antibodies, hemagglutination inhibition, and serum neutralization are for type-specific antibodies. FA staining is fast but needs fluorescent microcopy and specific skills. These tests are not ideal for easy performance in military environments. Immunochromatography color strips were developed for the rapid testing of respiratory pathogens, but are based on visual inspection and have been reported to have ***poor sensitivity*** [7-9]. Compare to the cell culture, the sensitivity of adenovirus test is < 73%, and *strep* A is test is less than 85% [7-9]. Therefore the Navy is looking for a simple, rapid, and sensitive point-of-care testing (POCT) tool, which can identify emerging strains of pathogens of interest to the military. ***Unfortunately, for many emerging pathogens, rapid and sensitive diagnostic tools are still not available.***

2.0 THE SOLUTION

Maxwell Sensors Inc. (MSI) ***proposed to develop a non-invasive pharyngeal pathogen sensor (PPS) system: based on an array of one-step fluorescence immunoassay (FIA) strips for respiratory pathogens panel detection and identification.*** The FIA strip disk provides operational simplicity, high sensitivity and rapid diagnostics for an array of respiratory diseases. Unlike conventional membrane-based immunoassays, which have poor sensitivity [7,8], the PPS system produces accurate and sensitive results in a compact hand-held unit. The self-calibrating FIA technique compensates for membrane variations and allows the PPS system to provide accurate and sensitive results. More importantly the proposed PPS POCT hand-held system is robust, such that it may be taken into the military field environment without compromising test accuracy. The operation procedure is very simple and easy enough to be used by personnel with no medical training (see Figure 1-1). A throat swab is obtained from the subject; then the swab sample is inserted into a plastic tube with an extraction buffer solution. The tube is squeezed to deliver the specimen into the strip disk. ***After inserting into the palm reader, the reader automatically shows the respiratory pathogen panel (RPP) results within 10 minutes.***

The PPS system integrates an array of fluorescence membrane strips, and a palm reader with optoelectronics for easy, rapid, and sensitive detection. ***Fluorescent immunoassay is one of the most sensitive direct optical measurements.*** When the specimen is deposited into the sample well, the sample is distributed into the array of membrane strips (~ 4 mm x 40 mm) and migrates down to the strips by way of capillary action. The antigen (e.g. adenovirus) migrates to the membrane and binds to the labeled monoclonal antibody (Ab*) to form Ag-Ab*, which is then captured by the immobilized anti-adenovirus and forms (Ab-Ag-Ab*) at the capture zone. At the same time the control beads, containing the known amount of anti-goat antibody, are trapped by the immobilized goat IgG at the control zone and form Ab*-Ag, while unreacted material is transferred to an absorbent pad at the end of the strips. The fluorescent signals from the capture zone is measured and normalized to that from control zone; the normalized signal is correlated to

the analyte concentration. The system's analytical sensitivity and linearity, which are based on the slope of the analyte concentration - fluorescence response curve generated from a series of samples with known analyte concentrations, are used as a calibration curve. **Due to FIA-based tests have low detection limit, the PPS system will dramatically improve the POCT sensitivity and accuracy.**

The highlights of MSI's proposed PPS system include:

1. A easy to perform, non-invasive (throat swab) system for rapid (< 10 minutes) detection
2. Array of strips for ***simultaneously multiple (6 - 10) pathogens detection***
3. The first device to use FIA Strips System for high sensitivity and quantitative respiratory pathogens measurements
4. The method is very sensitive and highly specific
5. Low cost strip test ($1- $2) and PPS ($800 - $1,200) system
6. Compact computerized portable unit with advanced optoelectronics for military and POCT uses.

Rapid Respiratory Testing:
Finding the cause of viral and bacterial infections for earlier treatment.

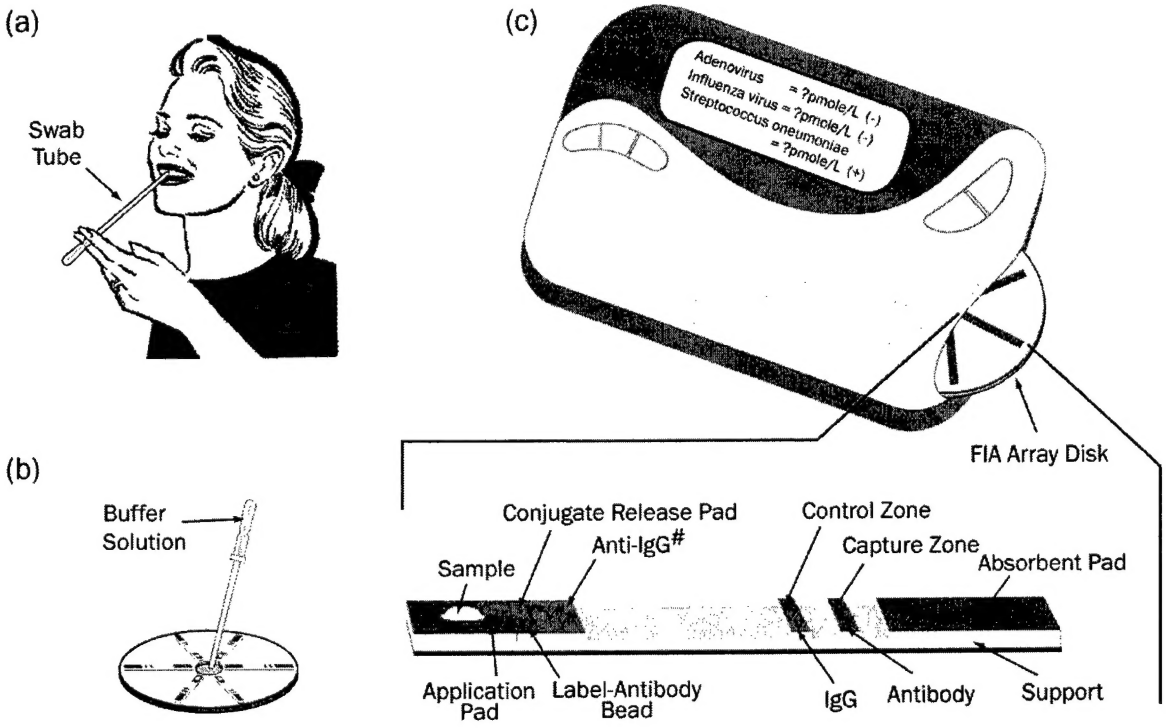

Figure 1-1. The proposed portable pharyngeal pathogen sensor (PPS) system, utilizes a disposable disk (3" in diameter) with an array of analyte-specific FIA membrane strips (4mm x 40mm) for respiratory pathogens detection and identification. After the sample is collected into the sample cavity, the sample migrates to each strip, reacts with label-antibody (or antigen) conjugates, is captured by a specific antibody, and is measured through optical means. The PPS is easy to use, robust, inexpensive, and yet highly sensitive. (a) A throat swab is obtained, (b) Once the throat swab is obtained from the subject, the swab sample is then inserted into a plastic tube with an extraction buffer solution. The tube is squeezed to deliver the specimen into the strip disk. (c) After inserting the disk into the palm reader, the reader will automatically show the results within 10 minutes.

3.0 PHASE I RESULTS

The objectives of Phase I revolved around the ability to demonstrate the technical feasibility of using the proposed FIA system for clinical diagnostic applications. During the phase I project the Principal Investigator, Dr. Winston Ho, (498 hours) was responsible for the design and construction of array strip and optoelectronic system for fluorescent detection. Dr. Ted Ou (438 hours) was responsible for bioassays and material preparation. Engineers, Markus Tarin (250 hours) and Peter Low (48 hours) designed and fabricated the optical system and electronics for the signal interface, collection, and processing. All of the Phase I objectives below have been achieved.

Objective 1. ***Developed FIA-based membrane strip for sensitive assay***
Objective 2. ***Investigated and selected immuno-specific agents and materials***
Objective 3. ***Constructed hand-held pharyngeal pathogen sensor (PPS) system***
Objective 4. ***Performed bioassay, obtained assay linearity, detection limits: FITC (0.1 picomole or 100-500 beads), clinical analytes: human IgG (0.1ng), streptococcus pyogenes (10^4 organism), adenovirus (0.1ng), influenza A (0.5ng), coefficient of variation (CV) (8%), and a total assay time of 10 minutes. These sensitivities are one to two orders better than that of the current POCT systems.***
Objective 5 ***Evaluated Commercial Potential and obtained a letter of interest with a funding commitment of $200,000.***

The Phase I results successfully demonstrated that it is possible to use FIA strip as a platform to deliver a small quantity of sample for multi-pathogen detection, and yet provide a rapid and high sensitivity for analyte detection. The detailed Phase I work is described below.

3.1 Task 1. Prepared Materials and Reagents

The potential targets are *adenovirus, influenza virus, Mycoplasma pneumoniae, Chlamydia pneumoniae, Streptococcus pneumoniae, Bordetella pertussis,* and *Streptococcus pyogenes*. In Phase I we focus on the detection of *adenovirus, influenza A virus, and Streptococcus pyogenes* for feasibility studies. The assay protocol is based on the lateral flow fluoro-immunoassay with a sandwiched structure. For each target antigen, we need to prepare a fluorescent labeled monoclonal antibody conjugate for signal detection and polyclonal antibody as a capture. Here we listed the properties of these materials.

**** Streptococcus pyogenes***

Product:	*Streptococcus pyogenes (gp. A)*
Vendor & Catalog #:	Quidel: heat-inactivated Positive control sample
Contamination:	May contain cellular debris
Form:	Liquid in sodium nitrite and acetic acid
Product:	Monoclonal anti- *Streptococcus A*
Vendor & Catalog #:	Seradyn MIS0102
Host:	Mouse spleen cell
Immunogen:	Strep group A carbohydrate
Subclass:	IgG
Form:	Liquid in potassium phosphate buffer, purified by DEAE, pH 7.2
Product:	Polyclonal anti- *Streptococcus A*
Vendor & Catalog #:	Lee Lab. 210490
Host:	Goat
Cross reactivity:	It does not react with other Strep groups
Form:	Liquid in phospate buffer, purified by protein A, pH 7.2

*** Influenza**

Product:	Influenza A virus
Vendor & Catalog #:	Fitzgerald 30-A150
Grade:	ELISA
Contamination:	No reactivity with Influenza B
Form:	Liquid in PBS buffer

Product:	Monoclonal anti-Influenza A virus
Vendor & Catalog #:	Fitzgerald 10-150
Host:	Mouse
Immunogen:	Influenza A from A/Texas strain
Subclass:	IgG2
Affinity constant:	$1.2x10^{10}$L/M
Cross reactivity:	Reacts with all subtypes
Form:	Liquid in PBS buffer, purified by protein A, pH 7.2

Product:	Polyclonal anti-Influenza A virus
Vendor & Catalog #:	Fitzgerald 20-IG24
Host:	Goat
ImmuIgG2:	Influenza A, H3N2 stain
Affinity constant:	$1.0x10^{10}$L/M
Cross reactivity:	It does not react to Hep-2 cells
Form:	Liquid in PBS buffer, purified by protein A, pH 7.2

*** *Adenovirus***

Product:	Adenovirus
Vendor & Catalog #:	Fitzgerald 30-AA02
Grade:	ELISA
Contamination:	May contain cellular debris
Form:	Liquid in MEM buffer

Product:	Monoclonal anti-Adenovirus
Vendor & Catalog #:	OEM Concept M2-V01
Host:	Mouse
Immunogen:	Hexon antigen of human adenovirus
Subclass:	IgG2a
Cross reactivity:	Reacts with all subtypes
Form:	Liquid in PBS buffer, purified by protein A, pH 7.4

Product:	Polyclonal anti-Adenovirus
Vendor & Catalog #:	OEM Concept G4-V01
Host:	Goat
Immunogen:	Hexon form adenovirus type 2
Cross reactivity:	It does not react with Para1-3, infl. A & B or RSV
Form:	Liquid in phosphate buffer, purified by protein A, pH 7.2

*** Latex beads, Chemicals, and Materials**

1. FITC-Microparticles: Magsphere CMY2157, 0.332 micron, 10% solid
2. Membrane: S&S 8micron pore size, Millipore 8 micron pore size
3. BSA-PBS-Tween: 1%BSA-PBS-0.1% Tween 20
4. MES stock buffer, 0.5M, pH 6.1, Sigma 69892

5. EDAC: Sigma E 6383
6. Storage buffer: Tris 0.05M, sucrose 5%, Tween 20 0.1%
7. Absorbent paper: Ahlstrom 222
8. Glass fiber: Ahlstrom 8980
9. Vinyl backing: G & L 187
10. FITC: Sigma Fluorescein isothiocyanate, F 7250
11. Conjugate buffer: Carbonate-bicarbonate buffer, 0.1M, pH 9.0
12. Gel filtration column: 10ml Sephadex G25-150,

3.2. Task 2. Prepared Membrane Strip and Antibody Application

3.2.1 Membrane-immobilized antibody

Antibody capture was immobilized on the membrane based on the following method

1. Dilute the capture antibody to 2-5 mg/ml in phosphate-buffered saline (PBS).
2. Use Line-Drawer (Fig. 3-1) to apply the antibody onto the membrane.
3. Dry the membrane for 1-2 hours at ambient temperature.
4. Block membrane by immersion in the BSA-PBS-Tween blocking solution for 30 minutes.
5. Dry the membrane for 30 minutes at 37C. Store at 2-8C.

The line-drawer, Model 3000 for clinical and pilot-plant production, was built by Nouvas Bio/Medical Automation. This station has two head spray dispensing units that easily and conveniently dispense different types of solutions onto a membrane surface. The direction, length, and width of the lines are adjustable. The volume dispensed by the unit's air-brushes can range from less than 0.2µl to greater than 5.0µl per centimeter. The spotting patterns can also consist of small (0.8 mm diameter) dots, to lines of various lengths and widths.

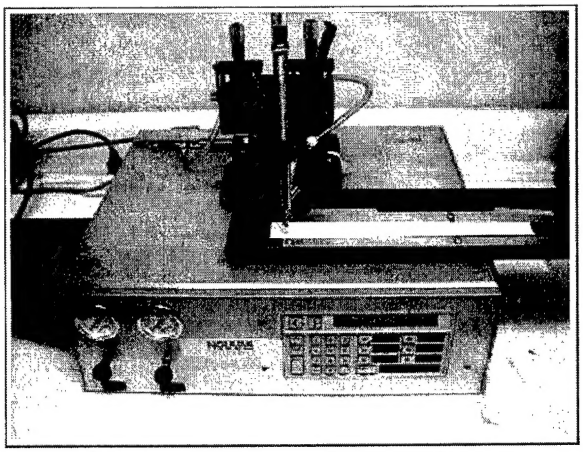

Fig. 3-1. The line-drawer (Model 3000, Nouvas Bio/Medical Automation) was used to apply the capture antibody on the membrane.

3.2.2 Tagged label particle (latex bead)-antibody

1. Prepare the binding reaction in the following order:
   100µl 0.5M MES stock buffer
   Water to make 2.0 ml in the final volume

50-100µl of 10.0% stock FITC-microparticles
Antibody stock solution to 0.2-1.0 mg/ml
Mix at room temperature for 5-10 minutes

2. Prepare the EDAC at 10 mg/ml before use. Add EDAC at 0.1-02 ml
3. Mix the binding solution for one hour at ambient temperature.
4. Centrifuge microparticles and remove the supernatant.
5. Resuspend microparticle pellets by ultrasonication. Wash with 50mM MES buffer. Repeat one more time.
6. Resuspend final microparticle pellets in 1.94 ml of storage buffer.
7. 5-20µl of tagged particle-antibody was used per test, either directly mixed with a sample or dried on a glass fiber filter.
8. The formation of fluorophore bead-antibody was monitored by the absorption spectroscopy (see Fig. 3-2).

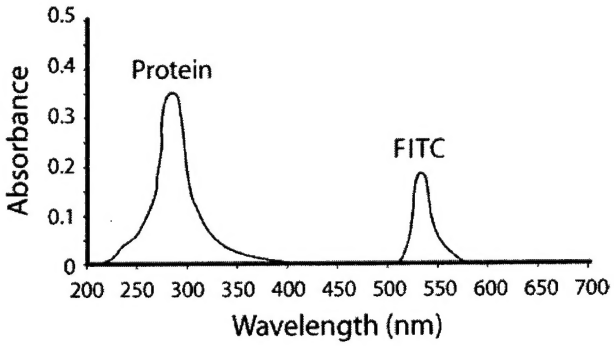

Fig. 3-2. The confirmation of FITC-antibody formation is monitored by the spectroscopy. Peaks at 280nm and 530 nm are the spectral nature of proteins and FITC, respectively.

3.2.3. Tagged fluorophore-antibody Preparation

1. Dialyze antibody into conjugate buffer at 1 mg/ml.
2. Dissolve FITC in a conjugate buffer at 1 mg/ml.
3. Add 20µl of FITC solution to 1ml of the antibody solution while stirring.
4. Incubate for 1 hour at ambient temperature in the dark.
5. Use a desalting column to separate unreacted FITC from the conjugated antibody.
6. Collect the FITC-antibody fractions and add BSA-PBS-sucrose stock solution to obtain a final concentration of 1 % BSA and 5 % sucrose.
7. Load the FITC-antibody onto the glass fiber filter in a desirable amount and dry at 37C for 1 hour.

3.2.4 Prepare Lateral Flow FIA Strips

The strip consists of four parts: the sample pad, the fluorescent-tagged antibody pad, membrane with binding antibody and the absorbent pad. To assay, sample is added to one end of the strip, the sample pad (see Fig. 3-3). Through the capillary effect, the sample is moved along the flow path with the tagged antibodies. The tagged antibodies migrate with the analyte to the capture zone of membrane-immobilized antibody. Unreacted tagged antibodies migrate to the end of the strip. The absorbent pat at the distal end of the strip aids in drawing the sample through the strip. The tagged antibodies are prepared with FITC-microparticles or FITC dye. Although the signal

generated from the use of FITC-microparticles is higher than that of FITC dye, the preparation of FITC dye-antibodies has advantage for simplicity.

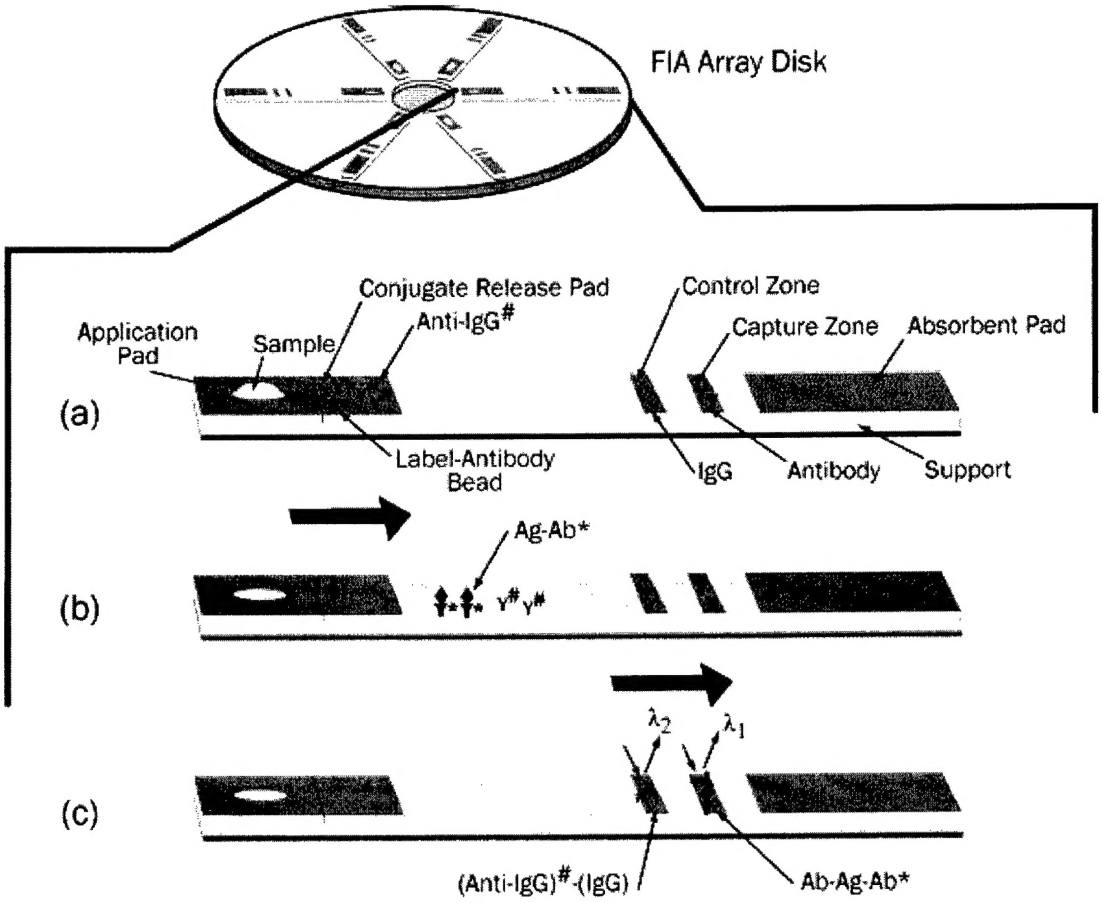

Fig. 3-3. (a) Lateral flow fluorescence sandwiched immunoassay for respiratory pathogen sample application (top). (b) Sample migration and analyte (Ag) binding with the label antibody to form (Ag-Ab*) (middle). (c) Fluorescent signals from (anti-control)-control at the control zone and analyte conjugates (Ab-Ag-Ab*) at the capture lines (bottom).

3.2.5 Assembled FIA cartridges

For Phase I feasibility study, single-analyte FIA strips were packaged with cartridges (Fig. 3-4)_ for easy handling. The assembling process is shown in the following.

1. Put membrane on vinyl backing.
2. Put absorbent pad at the distal end and overlap the membrane.
3. Put tagged pad overlap at the other end of the membrane.
4. Put the sample pad at the other end of the overlap for the tagged pad.
5 Cut the strips according to a desired width (0.4cm).
6. Put a strip in the cassette housing.

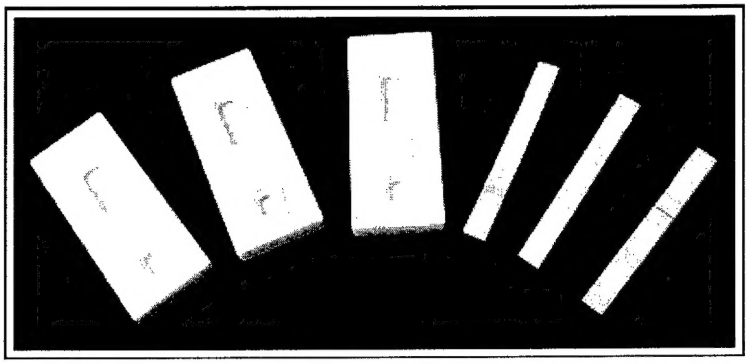

Fig. 3-4 The FIA-based *Streptococcus pyogenes* strips were assembled in a cassette housing in Phase I.

The idea of multi-analyte FIA strips has been demonstrated in the laboratory. An array of strips can be arranged in two configurations: FIA disk and linear arrays were used. With the disk array, multi-strips were placed on a rotational stage (Fig. 3-5). The rotational stage moved the strip to be tested under the optical probe.

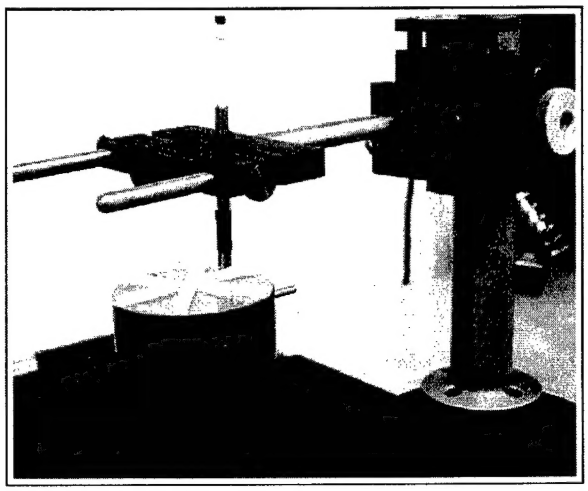

Fig. 3-5. Multi-strip (analyte) FIA disk was placed on a rotational stage.

3.3. Task 3. Constructed PPS Optoelectronic System

To monitor the excitation and fluorescence, a miniaturized spectrometer was used. High powered LEDs, blue (470nm), was used to excite the FITC fluorophore. Newly developed LEDs are inexpensive, have very stable output, and have a very long usable life. The light source is coupled to an optical fiber probe through an SMA connector that allows for coupling to fiber optics. A narrow band optical filter with a 10nm bandwidth was used to remove the tail of the excitation light source. This filter is critical to minimize the background signal and improve the detection limit of the system. The light source can be configured to operate in pulsed or continuous mode with software.

The bifurcated optical fiber was used as a probe for both illumination light delivery and fluorescent collection back to the detector. A bifurcated fiber with 1 (probe) × 2 (light source and detector) arms is highly suitable for this application. The fiber probe can be fabricated with a large number of optical fibers assembled with the same dimension as the capture line. Two probes, one for control and one for the target analyte, can also be installed directly on top of each sensing zone. The light source, optical fiber probe, and the detector (CCD) were assembled as a compact module (3"x3"x3") in Phase I. The fluorescence light was collected and filtered through a high pass filter (OG 530 from Rolyn Optics) to remove the excitation light. The distance and angle of the fiber probes and sample are also important to maximize the signal/noise ratio. Based on the current system configuration: a 2 mm distance is optimal for signal collection, and a 10 degree angle can significantly reduce the back collection of the excitation light source and allow us to obtain maximum fluorescence. The Phase I PPS system is designed based on miniaturized optical fiber probes to ensure the system's compactness, robustness, and reliability; all system components are integrated with solid hardware (see Figure 3-6).

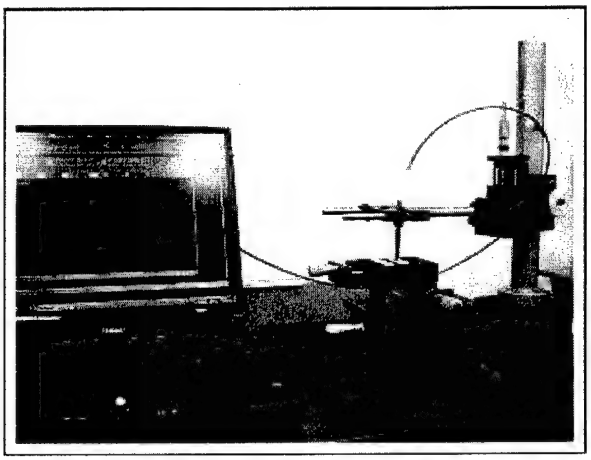

Figure 3-6. The photo shows the MSI PPS system with an optical fiber probe on top of the FIA strip. The optical probe coupled with a very compact light source and detection system is interfaced to a notebook computer. The software displays both fluorescence spectrum and intensity level.

3.4. Task 4. Perform Bioassay and Characterized PPS System

3.4.1 System Sensitivity Characterization

Different fluorophores have been developed for biological, biochemical, and clinical purposes. FITC and TRITC are the most frequently used probes for FIA (see Table 3-1). FITC absorbs at 480nm and fluoresces in the green region (visible to the eye), while TRITC absorbs green and generates red fluorescence. Cy5, Nd-benzoytrifluoroacetone and phthalocyanine have strong absorption at 630nm, thus they are suitable for excitation with a near-IR light source. FITC is our first choice for fluorescence assay experiments. We will investigate Cy5 in Phase II.

Table 3-1. Properties of Some Fluorescent Probes Used in FIA

Probe	λ abs/exc, nm	ε, L/mol	λ emis, nm	Quantum Yield, %
Fluorescein (FITC, DTAF)	492	7×10^4	520	0.85
Rhodamines RBITC TMRITC	550 550	1.2×10^4 1.2×10^4	585 580	0.70
Nd-benzoytrifluoroacetone	800		900, 1060, 1350	
Sulfoindocyanine (Cy5)	630, 670	2×10^5	685	0.7

The purpose of the experiment is to characterize the system's optical detection limit based on direct fluorescence measurement. To characterize PPS system performance, two tests: the FITC (fluorescein isothiocyanate) bead and FITC dye were performed. The fluorophore does not couple with protein.

* *FITC beads*

The FITC beads provide the primary amines of proteins to form the desired dye-protein conjugate. The absorption and fluorescence emission maximal of FITC-labeled protein is approximately 480 nm and 530 nm, respectively (See Fig. 3-7). The carboxylated PS latex bead solution, 10%, with different sizes 0.044μm, 0.104μm, and 0.332μm, were purchased from Magsphere Inc. (CA). Latex beads were diluted in a MOPS buffer (0.1M, Tween-20 0.1%, pH 4-6.0). Although different sizes of latex beads were used, no measurable signal difference was observed. The experiments were performed by dilution of the FITC bead stock solution (10M - 50 M beads/ml). The dilutions (0.1%, 0.01%, and 0.001%) were prepared to make up the concentrations for system tests. The FITC beads were directly apply onto the capture line of the strip. The typical spot sizes were 1 - 2 mm in width. The typical FITC bead concentration used in clinical testing is 0.1 % or 0.01% 10,000 - 5,000 beads. Excellent signal/noise ratios were obtained on Sample 3 with a dilution of 0.001% or equivalent to a concentration of 100-500 beads/ml (Fig. 3-8). **Therefore, the system achieved a detection limit one to two orders of magnitude better than the typical clinical ranges.**

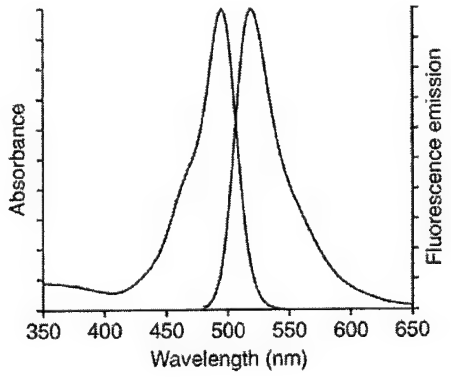

Figure 3-7. The absorption and fluorescent spectra of FITC beads. FITC beads are used to report the positive identification of analytes and provide the fluorescence signal for detection and quantification.

The collected signals obtained from the detector were interfaced with PCs via A/D converters. The system and software are capable of performing real time subtraction of background signals or reference signals. The exposure time was set at 100-300 ms. Sample signals and background signals increase as a function of the integration time.

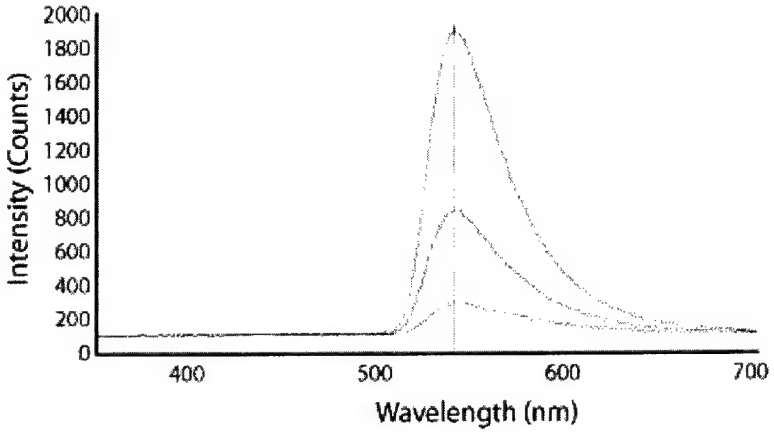

Fig. 3-8. The data of fluorescent signal versus various dilutions of FITC latex beads from the stock solution: top curve 0.1 % ($5x10^4$ beads); middle curve 0.01 % ($5x10^3$ beads) ; and bottom curve 0.001 % ($5x10^2$ beads) on membrane strips.

* *FITC dye*

FITC dye experiments were performed by directly applying different concentrations of FITC solution onto the test strip. The concentration of the FITC ranged from 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} molar, and the application volume was 10µl. Different amounts of FITC solutions, 0.1, 1.0, 10, and 100picomole, were spotted on the capture line. Figure 3-9 shows the system's sensitivity by measuring the fluorescence generated from FITC dye on a strip. The significance of this implication is that if an antibody and dye molecule ratio is 1:1, then a 0.1picomole analyte is detectable. **This data shows an excellent detection limit of 0.1picomole was obtained with the system.**

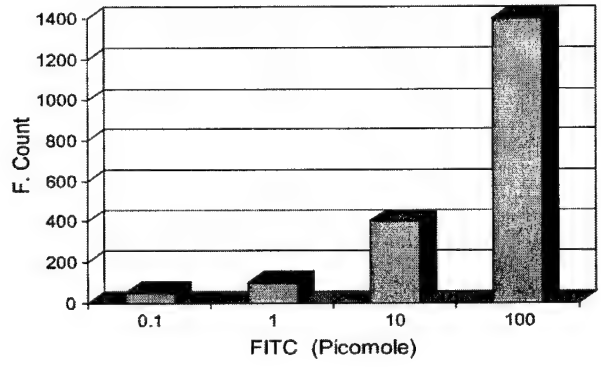

Figure 3-9. The data of various dilutions of FITC: Sample 1. 0.1 picomole, 2. 1.0 picomole, 3, 10 picomole, and 4. 100 picomole, characterized in the strips by the PPS system.

3.4.2 Control Experiments with IgG

The PPS system is self-calibrating, a control line is used to correct for membrane variability and provides an internal validation of the assay (Table 3-1). To establish a quantitative reading, the system calculates the ratio between the intensities detected at the capture and control lines. The control analyte, anti-IgG solution-FITC (50ng/ml) (Sigma, F9512, 2ml), was prepared in the PBS solution. IgG (Sigma, I4506, 10mg) was immobilized at the control line (10 μg/line). After 10 minutes of incubation, 20μl anti-IgG-FITC was captured by the IgG at the control line, and the fluorescence intensity was measured. **The experiments were repeated five times and the coefficient of variation, CV, was ~8%, which is much better than the 15%-40% CV in the conventional color strips (Fig. 3-10).** Therefore, the control line can be useful for signal calibration.

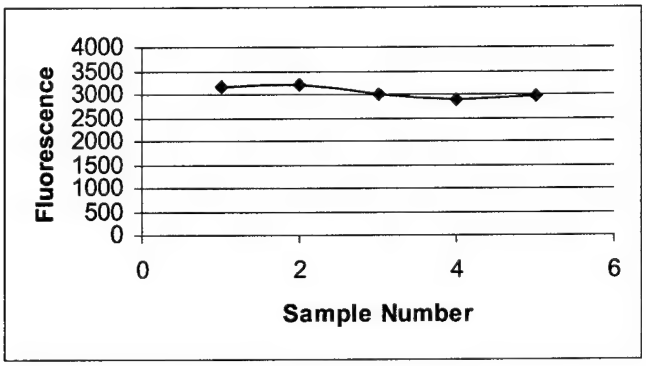

Fig. 3-10. The fairly constant intensity at control line can be used for signal calibration. The data shows the fluorescence intensity of the control sample, anti-IgG-FITC, at the control line. **The experiments were repeated five times and the coefficient of variation, CV, was ~8%, which is much better than the 15% - 40% CV in the conventional color strips**

3.4.3 Bioassay for Respiratory Pathogen Detection

3.4.3.1 *Streptococcus pyogenes Tests*

Monoclonal anti-*streptococcus* was conjugated to FITC-beads. This solution is applied to the conjugate release pad. Polyclonal anti-s*treptococcus* A was immobilized at the capture line of the membrane in a concentration of 10 μg/line. The *streptococcus* samples were prepared with dilutions on the standard control sample from Quidel Corp. Samples (or sample titers) (150μl) were loaded onto the sample pad. *Streptococcus* A binds to the antibody on the release pad and migrates along the membrane. These complexes are captured at the capture line. ***The total incubation time is 10 minutes.*** The fluorescence intensity at the capture line was measured in order to determine the concentration of streptococcus. Figure 3-11 shows the plot of fluorescence spectra for various analyte concentrations. The QuickVue test yield positive results with specimens containing $50x10^4$ microorganisms per test. The microbiological agent is negative for gp. B, C, D, E, F, and G. The PPS system's analytical sensitivity is based on the slope of the analyte titers ($1x10^4$, $10x10^4$, $20x10^4$, $35x10^4$, $50x10^4$ *Streptococcus pyogenes*) - fluorescence response curve, as shown in Fig. 3-12. Based on preliminary tests, we have achieved a sensitivity of detecting $1x10^4$ *Streptococcus pyogenes*. This sensitivity is 50X better than current rapid testing strips available on the market.

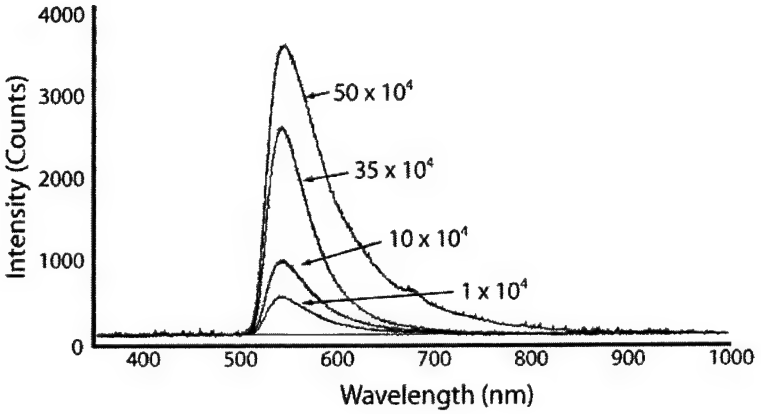

Fig. 3-11. Fluorescence spectra for various concentrations of *streptococcus pyogenes* tests. The data was plotted in the Fig. 3-12.

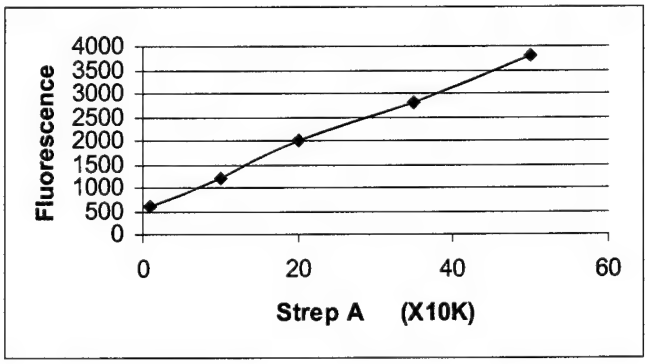

Fig. 3-12. The PPS system sensitivity analysis: fluorescence intensity as a function of the *Streptococcus pyogenes* concentration. ***The Phase I PPS have achieved a sensitivity of detecting 1x10⁴ Streptococcus pyogenes. This sensitivity is 50X better than current rapid testing strips available on the market.***

3.4.3.2 *Adenovirus Tests*

The samples were titer with a stock *adenovirus* (Fitzgerald 30-AA02, hexon from Adenovirus, Type 2)) viral agent solution with 5.0mg/ml protein concentration. It does not react with Parainfluenza 1,2, or 3, influenza A & B or RSV. Monoclonal anti-*adenovirus* was conjugated to FITC-beads. This solution is applied to the conjugate release pad. Polyclonal anti-a*adenovirus* was immobilized at the capture line of the membrane in a concentration of 10 µg/line. Samples (or sample titers) (150µl) were loaded onto the sample pad. *Adenovirus* binds to the antibody on the release pad and migrates along the membrane. These complexes are captured at the capture line. The fluorescence intensity at the capture line was measured in order to determine the concentration of *adenovirus*. The total incubation time is 10 minutes. The PPS system's analytical sensitivity is based on the slope of the analyte titers (0.1, 5.0, 20, 100, 200 ng *Adenovirus*) - fluorescence response curve, as shown in Fig. 3-13. ***Based on preliminary tests, we have achieved a detection limit of 0.1ng.***

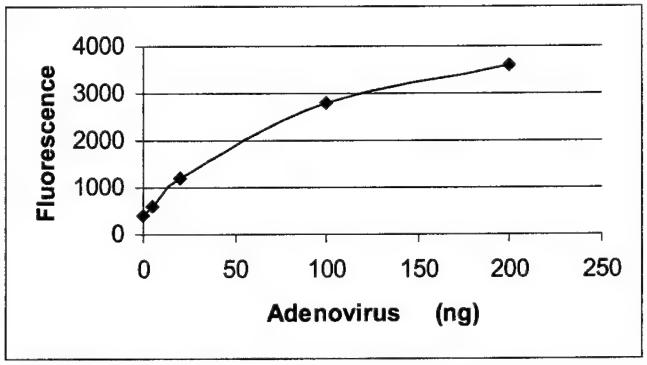

Fig. 3-13. The PPS system sensitivity analysis: fluorescence intensity as a function of the *adenovirus* concentration.

3.4.3.3 *Influenza A Tests*

The viral agent samples were prepared with dilutions on the *influenza A* (Fitzgerald 30-A150) stock solution (4.9mg/ml protein concentration). It does not react with Influenza B, RSV, adenovirus or Parainfluenza 1-3. Monoclonal anti-*influenza A* was conjugated to FITC-beads. This solution is applied to the conjugate release pad. Polyclonal anti-*influenza A* was immobilized at the capture line of the membrane in a concentration of 10 μg/line. *Influenza A* binds to the antibody on the release pad and migrates along the membrane. The PPS system's analytical sensitivity is based on the slope of the analyte titers (0.5, 5, 20, 50, 80ng) - fluorescence response curve, as shown in Fig. 3-14. Based on preliminary tests, we achieved a detection limit of 0.5ng *influenza A*.

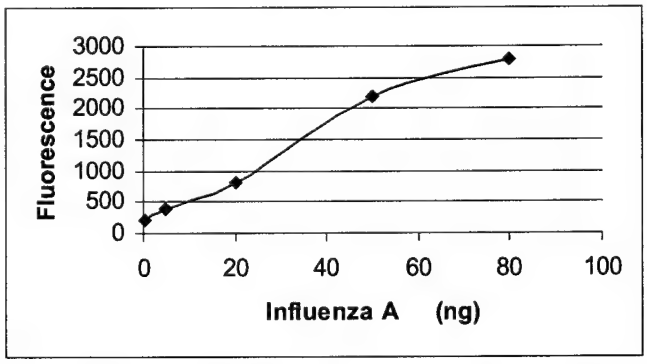

Fig. 3-14. The PPS system sensitivity analysis: fluorescence intensity as a function of the *influenza A* concentration.

3.5 Current Competing Technologies

The laboratory diagnosis of viral infections is usually accomplished by serology, culture isolation/confirmation and direct detection. The drawback to serological diagnosis is the delay and inconvenience of obtaining acute- and convalescent-phase sera. In addition, individuals may be incapable of mounting an adequate antibody response to respiratory viruses. Historically, culture isolation/confirmation was the standard method used by most clinical virology laboratories. Although it is not as rapid as direct detection from patient specimens, culture isolation/confirmation is still the most sensitive method for detecting viral respiratory pathogens. Direct detection of viral antigens in patient specimens allows rapid diagnosis and the possibility

of prompt antiviral treatment. Rapid diagnostic assays are commercially available from Biostar (*Strep* A OIA), Quidel (QuickVue -Influenza), Pacific Biotech, DPA, etc. These tests (Table 3-2) are either single pathogen (e.g. *Strep* A) tests, not sensitive enough, labor intensive, or require systems, which are not suitable for military hospitals and field application.

Table 3-2. Comparison of various methods for respiratory pathogen detection.[3-9]

	Culture	Fluorescence Staining	Enzyme IA	Color IA Strip	Fluorescence IA Strip
Operation Labor	Intensive	Intensive	Complex	Easy	**Easy**
Response Time	Days/ weeks	Hours	Hours	5 -10 minutes	**5 -10 minutes**
Sensitivity pg/ml (~ 10^{-15} mole/ml)	Very sensitive	Sensitive	Sensitive 0.1- 100	Poor 1,000-100,000	**Sensitive** 0.1 - 1,000
Sensitivity (Organisms)	1 -10^3 organism	10^3-10^4 organisms	10^3-10^4 organisms	10^5-10^7 organisms	**10^3-10^4 organisms**
Hardware	Incubation wares microscope	Microscope	Lamp, PMT	Visual/LED, photodiode	LED (LD), APD
System Cost Price	Expensive	Expensive > $5,000	Expensive > $6,000	Cheap $100 - $450 (if reader is used)	**Inexpensive** $800 - $1,200
System Size	Large	Large	Large	Small	**Small**

3.6 Phase I Summary

During Phase I of the project, we have developed FIA-based strip for sensitive assay, investigated and selected immuno-specific agents and materials, constructed hand-held pharyngeal pathogen sensor (PPS) system, characterized both strip and system with direct fluorophore tests, performed control sample (human IgG) tests and clinical analytes: *adenovirus, influenza A virus, and Streptococcus pyogenes* tests for feasibility studies. Table 3-3 summaries the data obtained from this Phase I program.

Table 3-3 Phase I assay analytical characteristics based on PPS testing.

	FITC	Human IgG (Fc)	*Streptococcus pyogenes*	*adenovirus*	*influenza A virus,*
Dynamic Range	0.1-100 picomole dye 10^2-10^5 beads	0 - 200 ng/ml	10^4 - 5x10^5	0 - 200 ng/ml	0 -80 ng/ml
Detection Limit	**0.1 picomole dye 100-500 beads**	**0.1 ng/ml**	**10^4 organisms**	**0.1 ng**	**0.5 ng**
Assay Linearity	Yes	Yes	Yes	Yes	Yes
Assay Time		**10 minutes**	**10 minutes**	**10 minutes**	**10 minutes**

The Phase I results conclude that we have successfully demonstrated feasibility by using fluorescence immunoassay strip platform for respiratory pathogen tests. The proposed PPS system not only achieves a rapid test (10 minutes), but also significantly more accurate (CV=8%), reliable, and sensitive than other current immunoassay-based diagnostic technologies by one to two orders of magnitudes. As a result, clinicians are provide with diagnostic information in minutes and, thus, may immediately begin proper therapy. This reduces the risks of complications and increase treatment effectiveness. Based on these technical feasibility findings, we are confident that we can launch into Phase II studies for further improvement.

3.7 References

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8. J. C. Dale, et. al. "Evaluation of two rapid antigen assays, Biostar strep A OIA and Pacific Biotech Card O.S. and culture for detection of group A streptococci in throat swabs", J. Clin. Microbiol. 33:3365 (1995).
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PHASE II PLAN

Phase I Solicitation Topic: OSD01-DHP06
Title: Rapid Diagnostics for Detection of Respiratory Pathogens

4.0 PHASE II TECHNICAL OBJECTIVES

Based on the Phase I foundation, the Phase II objective is to build and demonstrate a field-usable rapid infectious disease screening system that meets FDA requirements and allow reliable and effective detection of the respiratory diseases. The specific Phase II objectives established in order to reach this goal are detailed below.

Objective 1. ***Optimize membrane performance for quantitative assay***
The ionic strength and pH of the buffers and proteins, the protein-membrane affinity property, the density of fluorescent labels in the membrane still require optimization. This objective is to achieve an optimal result by performing series of membrane characterizations, optical characterizations, and comparison studies.

Objective 2. ***Refine Immuno-specificity for respiratory pathogens***
Infectious agents usually have more than one epitope (antigenic site). These epitopes have specific and non-specific binding for various proteins, which affect the sensitivity of the detection. Therefore, the selection of conjugated and captured antibodies for each target analyte is critical to the specificity. A series of tests based on known mixtures of analytes, groups, or strains will be conducted for specificity and sensitivity tests.

Objective 3. ***Complete PPS system integration***
The PPS laboratory system includes optical components, light sources, bifurcated optical fibers, holographic filters, and detector will be integrated into a prototype systems. To ensure the system's compactness, robustness, and reliability, all system components will be integrated with solid connections.

Objective 4. ***Increase the number of pathogen tests***
The number of respiratory pathogen test will be expanded from three in Phase I to six or seven in Phase II. The objective is to simultaneously screen for adenovirus, influenza virus, Mycoplasma pneumoniae, Chlamydia pneumoniae, Streptococcus pneumoniae, Bordetella pertussis, and Streptococcus pyogenes on a single FIA disk.

Objective 5. ***Perform clinical samples tests and characterize system***
The system will be tested and characterized based on the clinical samples. Both reactive and non-reactive samples, N = 50 - 100, for each pathogen will be used to test system performance. The comparison study will be performed using an FDA-approved system and MSI's PPS system. The correlation coefficient (γ) and coefficient of variation (CV) will be determined.

Objective 6. ***Develop user-friendly system suitable for operation in the field***
The PPS system will be designed to meet field use requirements. These requirements (such as compactness, light weight, easy operation, logistics, and reliability) are critical for use in the emergency medical environment. System connectivity via internet or wireless to the hospital information system will be investigated and developed.

5.0 PHASE II WORK PLAN

The main thrust of the research and development plan is to bring MSI's proposed method of rapid screening to the level of a prototype system and, in Phase III, to a commercial product. The Phase II project will build upon the solid Phase I foundation and extend the work to develop a PPS that can screen for the emerging respiratory pathogens, using the selected format to verify the required sensitivity and specificity. The following tasks will be performed and investigated during 24 months of the Phase II project.

5.1 Optimize Membrane and Protein Binding

The sensitivity of the assay depends on a number of factors: properties of the membrane, the binding density of antigens and antibodies at the capture line, the amounts of labeled fluorophores present in the conjugate release pad, and the amount of fluorophores attached to the antibody. The amount of fluorescence detected at the capture line is a linear function of the amount of antigen on the capture line; the proportionality constant is dependent on the procedures used to immobilize antibodies on the membrane. There are three ways that the application buffer can be adjusted to alter protein binding: 1. ionic strength, 2. pH, and 3. co-precipitation agent. In order to optimize protein binding to the membrane, MSI will experiment with various application buffer solutions.

5.2 Complete PPS System Integration

In Phase I, fluorescence signal was carried through an optical fiber into a spectrometer and CCD for spectral analysis. Once the fluorescence wavelength is known, the spectral grating and CCD will not be required. Instead, we will use a band-pass filter that has a sharp cut-off and a high optical density (O.D.= 4) to filter the wavelength. There are two methods can be used for monitoring the fluorescent spots from multiple strips. The first method (used in Phase I) is using optical fibers with no moving part. The bifurcated optical fiber provides both illumination light delivery and fluorescent collection back to the detector. Each fiber probe is associated with each strip. Using this configuration, signals can be collected from multiple strips simultaneously. The second method is using a rotational stage. A rotational stage will rotate and position the strip under the light source and detector for fluorescence measurement. In this case, a single set of light source and detector will be sufficient. We will investigate the trade-off of the two methods from performance, cost, and package requirement. The analog signal will be fed into the analog-to-digital converter, and then to the microprocessor for signal processing. The microprocessor will display the test results on the LCD screen.

5.3 Extend to Other Pathogens Assays

During Phase I, we investigated the technical feasibility by testing *adenovirus, influenza A virus, and Streptococcus pyogenes* tests. In Phase II we will extend the tests to other pathogens, such as *Mycoplasma pneumoniae, Chlamydia pneumoniae, Bordetella pertussis,* and *Streptococcus pyogenes*. Various resources for specific analyte tests have been identified and will be addressed in the formal Phase II proposal.

5.4 Bioassays Using Clinical Samples

The PPS for rapid diagnostic tests will be evaluated with clinical samples. The samples are from patients who are diagnosed as having pharyngitis, exudative tonsillitis, and pharyngoconjunctival fever. Both reactive (positive) and non-reactive samples for each analyte will be tested. The reactive sample (positive) will be titer by spiking non-reactive sample with various concentrations of analyte stock solution. Various analyte concentrations cover the clinical calibration range will be prepared.

Pharyngeal swabs will be obtained with two cotton tips. One swab will be inoculated directly into a cell culture bottle for pathogen isolation, and the other will be placed in 500µl of 10mM

Tris-HCl (pH 8.0)-1mM EDTA for the FIA tests. For the one-step PPS assay, 150µl of a pharyngeal swab specimen is transferred to the sample port on FIA strips. The specimen migrates via capillary action along the membrane. Samples will be obtained from Fitzgerald Industries International (MA) and BioSample (FL). Both companies are FDA certified laboratories and are FDA licensed facilities. The variety of sample pools will be tested for both specificity and matrix effect. The number of tests will be increased in order to obtain statistically significant results on sensitivity, accuracy, specificity, and response time. The false-positive and false-negative (%) reading will be determined based on the comparison studies with culture tests. The system is expected to perform approximately 50 tests per analytes; therefore, the total number of tests during Phase II is approximately 400-600.

5.5 Multi-analyte Respiratory Pathogen Panel (RPP) Testing

In practical applications, it is preferable to test more than one analyte. If each FIA strip is designed for one analyte test, it will require the clinician to deliver the sample to several strips. This is not very convenient. In Phase I we focus on the detection of *adenovirus, influenza A virus, and Streptococcus pyogenes* for feasibility studies. In Phase II, in addition to single analyte tests, PPS will be used to test *adenovirus, influenza virus, Mycoplasma pneumoniae, Chlamydia pneumoniae, Streptococcus pneumoniae, Bordetella pertussis,* and *Streptococcus pyogenes* on a single FIA Respiratory Pathogen Panel (RPP) disk (see Fig. 5-1). The multi-analyte RPP disk will be fabricated, and tested.

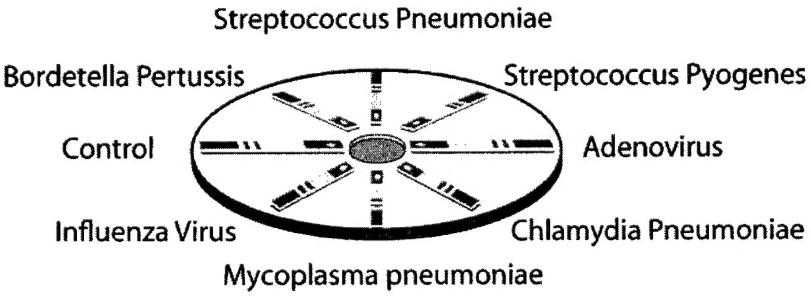

Fig. 5-1. The FIA-based Respiratory Pathogen Panel (RPP) disk will be used to screening the multiple respiratory pathogens simultaneously.

5.6 Characterize and Evaluate System Performance

In order to obtain the statistical values, the number of tests will be increased. An assessment of the accuracy of the results will be obtained for each assay. Both intra- and inter-CV will be determined based on repeated experiments. All data will be calibrated with the standard sample and background signal. An analysis of variance will be performed on the data, and the intra- and inter-coefficient of variation (%CV) will be determined. CV is equal to $(s/\bar{x})$ x 100, where s is the sample standard deviation and $\bar{x}$ is the sample mean. The system's analytical sensitivity is related to the slope of the concentration-response curve based on a series of samples with known analyte concentrations. The calibration curve provides the equation for data extraction. Once we have a reliable calibration curve for our system, the correlation between our system and other commercial system will be compared. Although some of the antigens have been tested for cross-reactivity by vendors. Due to the large number of bacteria and virus, more extensive cross-reactivity tests will be performed.

5.7 System Connectivity

In addition to providing rapid testing results, it is important to have the data connectivity form the field or remote sites to the military hospital information systems. The architecture proposed for point-of-care connectivity by the Connectivity Industry Consortium (CIC) will be adapted for

our current application. The CIC identified two architectures to employ standardization in order to simplify POCT connectivity: a Device Interface and an EDI Interface. The lower layer of the Device Interface is built on the IEEE 1073.3.2 standard, also known as the Medical Information Bus. During this task, MSI will follow the CIC guidelines and design a platform, which will be easy for future device adaptability.

6.0 COMMERCIAL APPLICATION AND ANTICIPATED BENEFITS

With the availability of antiviral therapy for some viruses, determination of a viral etiology for respiratory infections is important. Early and appropriate use of effective antiviral therapy can decrease morbidity and mortality associated with lower respiratory tract viral infections. Respiratory diseases cause significant morbidity and mortality, especially in young children and older adults. About 75 percent of acute respiratory illnesses are caused by viruses. Influenza A and B viruses could cause severe respiratory illness ("The Flu") and result in an average of 20,000 deaths per year in the U.S. alone. Physicians request virology services, because nearly half the ill children seen by the primary care physicians have acute respiratory ailments. The decision to use antiviral agents or antibiotics can only be made on clinical and epidemiological grounds, if viral diagnostic services are not available. Such treatment decisions are problematic because there is a significant overlap in the clinical syndromes caused by different infectious organisms. Therefore, a rapid diagnostic tool , which is sensitive and specific, is urgently needed.

7.0 TRANSITION PLANS

MSI will continue to consult with a Navy technical monitor and the researchers at the Naval Health Research Center to evaluate the proposed technology. Feedback will be used to optimize system performance and to develop system specifications for selected practical applications. Sources of Phase II & Phase III guidance and development funding will be identified early in the project and will be drawn from on a continuing basis. At the end of the project a complete report will be submitted, detailing the research completed and the results obtained during the course of the project. This report will also contain an evaluation of the PPS's technical merits and commercial prospects. It will also provide a synopsis of the potential applications for such devices. The success of the Phase II project will lay the foundation for successful commercialization in Phase III. A PPS prototype will be delivered to the Navy when Phase II concludes. MSI has a high interest in developing the Phase III program through internal funding, and joint development with private investors.

8.0 MATCHING DOLLARS

TECO Diagnostics (Anaheim, CA) manufactures and distributes one-step technology tests, immuno-diagnostics, hematology, urine chemistry reagent strips, drug adulteration strips, reagents, and clinical chemistry, including instruments to professional laboratories and hospitals around the world. Today the company has become internationally known as one of the leading manufacturers in the medical device industry, especially in the field of one-step reagent strips. The company is also known as a worldwide provider of high quality clinical diagnostic reagents and test kits. The commercial revenue of TECO Diagnostics is $25M as of the year 2001. TECO Diagnostics has agreed to provide **$200,000** to facilitate an agreement to negotiate a license for commercialization and distribution rights of the products. **A letter of interest from Dr. K.C Chen, the Founder and President of TECO Diagnostics is attached.**

9.0 QUALIFICATIONS OF KEY PERSONNEL

Dr. Winston Z. Ho, the Principal Investigator, has been working on immunoassay strips, fluorescence sensors, microfluidics, optical fiber probes, and biomedical instruments for many years. During his career at Physical Optics Corporation, Intelligent Optical System and Maxwell Sensors Inc, he has developed innovative technologies and products for various applications. These innovative technologies include: the Cytostrate with holographic surface morphology for

cell adhesion, the multiple channel optical waveguide for immunosensors, a cancer diagnostic system for photodynamic diagnostics, a non-invasive lactate sensor, etc. He also developed the first optical waveguide sensor by using non-linear (Coherent anti-Stokes Raman) laser spectroscopy to monitor a trace amount of analytes. Dr. Ho has been published more than 50 times and holds 4 patents in the areas of optical sensing, biosensors, chemical sensors, and physical sensors. He will be responsible for the design and development of the PPS optoelectronic system, and coordinating the team's efforts. He will be assisted by the team members, and take the technical lead for the design, fabrication, and testing of the proposed system.

Dr. C. Ted Ou, has extensive experience in the design and development of assay reagents for immunology, clinical chemistry, membrane strips, & hematology, and scale-up product process development. Through his careers at Technicon Instrument, Nygene Inc., Beckman Coulter Instruments and Abbott, Dr. Ou has commercial experience in developing latex immunoassays, EIA magnetic particles, enzyme immunoassays, and ferritin product development. When he was at Nygene Inc. (1987) he made a breakthrough contribution to the development of a reagent, which led to a turning point for system automation. Dr. Ou will be responsible for bioassay and material preparation for the TFIA strips.

Electronic Engineers, **Mr. Markus Tarin,** has extensive experience in system design, modeling, and construction. Optical Engineer, **Mr. Peter Low,** is responsible for designed and fabricated the optical system and electronics for the signal interface, collection, and processing.

10. PROPOSED FACILITIES/EQUIPMENT

Located in Los Angeles, California, Maxwell Sensor Incorporation's laboratories are designed for the development, testing, and manufacturing of various biochemistry and chemical sensors. The biochemistry facility has equipment includes electronic testing equipment, oscilloscope, Dynex (MRX) luminometer, microscopes, spectrometer, Beckman centrifuge, pH meter, environmental chamber, pouch sealer, incubator/oven, air brush line drawer, analytical balance, vortex mixer, and a variety of measurement instrumentation for analysis and system testing. The optoelectronic laboratory is equipped with a full complement of optical elements, optoelectronics sources, electrical circuit design instruments, mechanical systems, position equipments, optical benches, detectors, oscilloscopes, digital meters, and spectral analysis instrumentation. Many computers are available for the data collection, signal calibration, and statistical analysis.

11. ESTIMATED COSTS

The estimated cost for the Phase II project is approximately $750,000. The details of the budget will be provided in the formal Phase II proposal.

12.0 Performance Schedule

The stated tasks will be performed in accordance with the schedule (24 months) will be shown in the formal Phase II proposal.

ISO 9001
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TECO DIAGNOSTICS

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July 16, 2002

Dr. Winston Ho
Maxwell Sensors Inc.
10020 Pioneer Blvd, #103
Santa Fe Springs, CA 90670

Dear Dr. Ho:

Further to our recent meeting, TECO Diagnostics is desirous of commercializing your Chemiluminescence Biochip for cardiac markers tests. TECO Diagnostics manufactures and distributes one-step technology tests, immuno-diagnostics, hematology, urine chemistry reagent strips, drug adulteration strips, reagents, and clinical chemistry, including instruments to professional laboratories and hospitals around the world. Our commitment is contingent upon

(1) Maxwell Sensors Inc. receiving a Phase II grant from the National Institute of Health,
(2) the Phase II research achieving its technical objectives, and
(3) the planned technology remaining competitive during the Phase II period.

Due to the significance of the technology and market potential, contingent upon meeting the above criteria, our company agrees to provide $200,000 for the Phase III commercialization efforts. This funding support letter is offered to facilitate an agreement with Maxwell Sensors Inc (MSI) to negotiate a license for commercialization and distribution rights of the products. I am excited in exploring the opportunities offered by your products.

Sincerely;

Dr. K. C. Chen

Dr. K.C. Chen
President